Heat Denaturation of Whey and Model Protein Systems

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Abstract

The purified crystalline milk proteins, α-lactalbumin and β-lactoglobulin, as well as whole acid wheys, were denatured by a standard heating method as a function of pH. The effect of various ionic and nonionic additives on increasing the amount of centrifugable precipitate was studied. Neither simple ionic substances nor polyelectrolytes had substantial general effect whereas disulfide reduction of some protein components of whey by added cysteine caused a large increase in the amount of protein precipitated. The implications of these findings are discussed.

Introduction

In our pollution-conscious age, any manufacturing process which produces large amounts of waste is a legitimate target for research toward abatement or utilization of these waste products. Such a process is the manufacture of cheese. Drying whey to recover its solids has many practical drawbacks, particularly the large amounts of energy required to evaporate the water. Removal of proteins by precipitation would be cheaper and better, though still requiring disposal or use of the remaining lactose. Many groups are studying incorporation of whey proteins into foods. We do not attempt to touch on problems of such formulations but give findings which may increase the degree or rate of protein recovery. Nor is the recovered protein useful only as human food; wheys stored or handled under less than sterile conditions may be precipitated and the protein content used in feeds. Disposal of the remaining deproteinized whey would then be less troublesome.

The various proteins in whey were shown by Larson and Rolleri (11) to be heat-denatured at different rates; the immune globulin fraction is denatured first followed by the serum albumin which, although not a milk protein per se, is always in bovine milk. β-Lactoglobulin is less rapidly affected under the same heating conditions while α-lactalbumin is the most resistant.

Denaturation of β-lactoglobulin has been studied (5, 11, 17, and others) most recently by Lyster (13), who found the kinetics of the reaction to be second order with respect to time over a small concentration range but only at the pH of skim milk. Kenkare et al. (9) and Guy et al. (7) have also studied the heat aggregation of whey and whey proteins with practical emphasis on factors which hinder protein denaturation and maximize retention of native soluble character.

Nielsen et al. (18) have calculated recently the interplay of time, temperature, pH, and concentration variables on precipitation of cheese whey proteins by heat. These workers agree that increased concentration of whole whey has a protective effect on proteins. In our research the difficulty and expense of concentrating whey is deliberately avoided since the desired condition is maximal denaturation/precipitation of whey protein(s).

Purified β-lactoglobulin B and α-lactalbumin (and cottage cheese whey itself) were investigated with various additives, and the denaturation reported is defined operationally as formation of a centrifugable precipitate with no molecular definition.

Materials and Methods

β-Lactoglobulin B and α-lactalbumin were prepared from the milk of homozygous animals in the Beltsville ARS herd by the technique of Aschaffenburg and Drewry (1). The proteins were recrystallized three to four times and stored as centrifuged slurries under toluene. Acid whey was prepared from fresh skim milk by acidification to pH 4.6 with lactic acid at 25 C and filtration from the casein curd through a folded filter paper. The filtrate was shell-frozen in a dry ice-acetone bath and stored at −10 C until used. One month of storage had no noticeable effect on subsequent heat stability. Several batches of commercially prepared acid whey were also examined; however, the amount of protein precipitable varied

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between batches and was generally lower than our laboratory preparation, which was used for all precipitation reported in the figures.

Collagen solutions were prepared in our laboratory by extraction of comminuted cattle hide with dilute propionic and benzoic acids, followed by neutralization with NaOH. Sodium hexametaphosphate was obtained from Calgon[2] Inc. and had a degree of polymerization of 5 to 9. Other reagents were analytical quality or better, and deionized water was used throughout. We measured pH on a Beckman Zeromatic pH meter before heating. Protein concentrations were measured in a Zeiss PMQII spectrophotometer with the following absorptivities: β-lactoglobulin 9.6 deciliters/g at 278 nm (20); and α-lactalbumin 21.0 deciliters/g at 280 nm (10). Solutions of β-lactoglobulin were dialyzed overnight at 4 C against the bulk solvent. Aliquots were adjusted to the desired pH with 1 M HCl or NaOH and diluted to a concentration of 1 ± .02 g/100 ml with dialysate before heating.

Precipitation was effected by immersing the sample (generally 2 ml) in a test tube into an 80 C water bath for 15 min, conditions that caused 50 to 60% precipitation at the most in dilute NaCl (5). The sample was then chilled to room temperature with cool water and centrifuged at 59,000 × g for 20 min (30,000 rpm in a 40 rotor in the Spinco Model L Ultracentrifuge). The supernatant absorbance was then measured at the appropriate wavelength. From the absorbances measured before and after heating, percent protein remaining in solution was calculated.

In the cases where the purified proteins were dialyzed against skim milk, certain corrections were necessary. The riboflavin of milk has a significant optical absorbance in the UV range and at times nonsedimentable turbidity occurs in the samples. Any such turbidity will cause a false absorption of light due to light scattering, which can be quite significant at UV wavelengths. A technique (see appendix) has been developed to correct these effects. It involves dialyzing a water-filled sack against skim milk, using this dialysate as an optical reference in the spectrophotometer, and calculating from absorbance measurements taken at 280, 345, and 450 nm. In this way we sidestep definitions of protein versus nonprotein nitrogen and achieve internal consistency in these dirty supernatant solutions. In the case of acid whey, we report the amount of light absorbing material in the supernatant as soluble protein, without being concerned at this time with its nature.

In the few experiments in which $FeCl_3$ was an additive, the results were analyzed only by micro Kjeldahl nitrogen since Fe (III) forms strongly colored complexes absorbing broadly at all the usable wavelengths.

Results and Discussion

When the standardized heating procedure is applied to β-lactoglobulin B in .01 M NaCl (Fig. 1), the bell-shaped precipitation curve is similar to the pH-solubility curve of this protein (6), with its minimum close to the isoelectric point of 5.28. When salt content is increased to .1 M (open circles), smaller proportions of the β-lactoglobulin are precipitated, and the minimum solubility occurs at a pH of about 6. Although it is not shown in the figure, .5 M NaCl inhibits precipitation over the entire pH range from 3.5 to 8.5 when the protein is heated 15 min at 80 C.

Calcium, on the other hand, has an entirely different effect. This doubly-charged cation, at a concentration of only .01 M ($CaCl_2$, .01 M ionic strength .03) causes almost complete precipitation of β-lactoglobulin when heating is done at pH greater than 5.7 (Fig. 1). A higher concentration (.07 M, ionic strength .21) shifts the steep portion of the curve upward a fraction of a pH unit, and complete precipitation occurs above about pH 6. (This

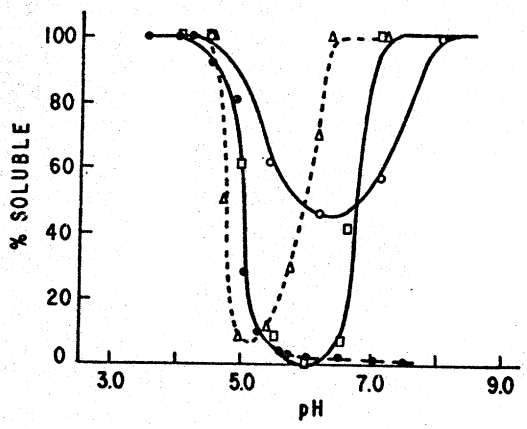

FIG. 1. Effect of solvents on heat-induced precipitation of β-lactoglobulin B. △, .01 M NaCl; ○, .1 M NaCl; ●, .01 M $CaCl_2$; □, dialyzed against skim milk.

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

concentration of calcium is close to the total calcium content of milk, both free and associated with casein.)

In addition to the soluble calcium content, whey contains 4 to 5% lactose and other dialyzable substances, including riboflavin, Na^+, K^+, Cl^-, and $PO_4^{\equiv}$ ions and significant amounts of soluble nitrogen, probably oligopeptides from uncompleted protein synthesis or from limited degradation of proteins subsequent to synthesis. This has been called the proteose-peptone fraction and is 2 to 6% of total milk nitrogen (4). To account for possible effects of these solvent components on the purified proteins, stock solutions of β-lactoglobulin B were dialyzed overnight against 600 ml of skim milk; aliquots then were subjected to the standard heat-precipitation procedure. The protein remaining soluble is shown in Fig. 1 as the lower solid line and open squares.

The pH of maximum precipitation is around 6, similar to .1 M NaCl solvent, but practically all the β-lactoglobulin is precipitated in the milk dialysate system. Other salts cause precipitation of whey proteins by formation of complexes which can be removed by filtration or sedimentation. Among these are ferric salts (2) and polyphosphates (3, 8, 14); both are effective at room temperatures, and both form reversible complexes. That is, the precipitants may be removed by dialysis and the protein recovered in a native soluble form. These substances might enhance heat coagulation of whey proteins and could be effective in lower concentrations than specified in the references. Furthermore, iron is a solution catalyst for disulfide interchange and might catalyze reduction-oxidation steps involving cystine-cysteine residues, which might be involved in the clotting phenomenon of heat-denatured proteins (5, 15, 19).

Fig. 2 shows results of these experiments. The addition of ferric chloride at 1 g/100 g of β-lactoglobulin (final concentration of $FeCl_3$ = 37 mM) followed by heating has little effect other than shifting the solubility minimum a little to the alkaline side and raising the trough. Evidently the amount of disulfide-interchange effect by iron is negligible, and the decrease in protein precipitated agrees with Block et al. (2), who state that their preformed iron-protein complex is stable to boiling without protein denaturation. The curve for dialyzed β-lactoglobulin in Fig. 1 is repeated here for comparison.

The data given by addition of sodium hexametaphosphate (HMP) are in Fig. 2. This reagent is highly effective as a precipitant for β-lactoglobulin in the milk-dialysate system over the pH range from 3.5 to 6 when it is added at the same concentration as $FeCl_3$ (.1 mg/ml of the 1% β-lactoglobulin). Fig. 3 shows that this concentration is more than necessary to cause satisfactory precipitation by heat. At a single pH, the concentration of HMP was reduced five times to .02 mg/ml and 98% of the protein still precipitated as compared with 50% at this same pH without HMP (see also Fig. 2). The molecular mechanism through which this precipitation occurs is only conjectural (14); nevertheless, it seems significant that 1/100 M calcium strongly aids precipitation from pH 6 to 8 and

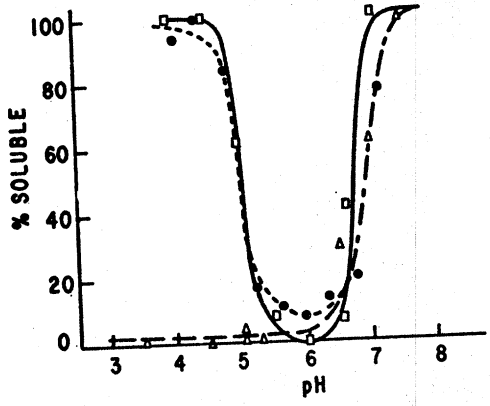

FIG. 2. Effect of added precipitating reagents on heat-induced denaturation of β-lactoglobulin B dialyzed against skim milk. □, dialyzed only; ●, $FeCl_3$ 1:100 added; △, hexametaphosphate 1:100 added.

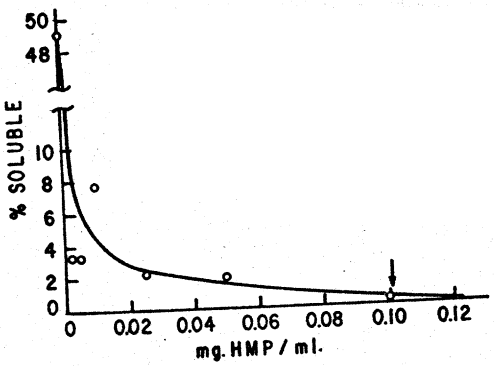

FIG. 3. Effect of sodium hexametaphosphate on heat-induced precipitation of β-lactoglobulin B dialyzed against skim milk and heated at pH 5.0. The arrow indicates the concentration HMP used in the experiments of Fig. 2.

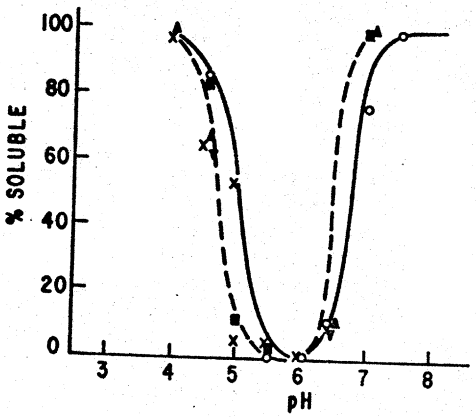

FIG. 4. Effect of various additives on heat-induced precipitation of β-lactoglobulin B dialyzed against skim milk. ○, made 2% in EtOH; ×, plus κ-carrageenan 1:20; solid points, cysteine HCl added: ▼, .013 M; ▲, .006 M; ■, .003 M. Solid line copied from Figure 1 (β-lactoglobulin, dialyzed, no additives).

HMP, a calcium sequestering agent, has the same effect from pH 3 to 6.

Several other additives were tried with negative results. Soluble hide collagen, added to the 1% β-lactoglobulin solution to a concentration of .2 mg/ml collagen before heating (solvent .1 M NaCl), gives a minimum solubility of 20% as contrasted to approximately 50% in this same solvent (Fig. 1) at essentially the same pH (i.e., 6.4 to 6.6) with little other change in curve shape.

Fig. 4 shows the effect of three other materials added to the milk-dialyzed β-lactoglobulin. Two percent by volume ethanol has no noticeable effect and kappa carrageenan (×'s) also has minimal effect or an effect obscured by salts and dialyzable materials of whey which, under our conditions, allow almost complete precipitation between pH 5 and 6.5.

Heat denaturation of whey proteins, β-lactoglobulin in particular, is accompanied by disulfide bond reactions (4, 5, 11, 15). (Also 19 for a further list of publications.) Morr and Josephson (15) have shown definitively that addition of sulfhydryl blocking agents hinders heat-induced whey protein aggregation. Addition of disulfide bond opening (reducing) agents might facilitate this aggregation since major covalent bonds holding the proteins in their native soluble configuration might be opened, and internal hydrophobic areas could then be brought into contact with water. However, in the original experiments, (Fig. 4) a two-fold stoichiometric excess of cysteine (Cys), (.43 mg cys-HCl/ml, 2.8 mM) causes no additional precipitation.

Fig. 5 shows results when purified α-lactalbumin was heated under our conditions. Due to the restricted solubility of this protein between pH 4 and 5, a .25% (wt/vol) solution was used. The figure shows that heating α-lactalbumin in .1 M NaCl gives a curve mimicking the room temperature pH-solubility curve (10). However, for this protein, calcium ion quantitatively interferes with precipitation over the entire pH range investigated. This should be contrasted with β-lactoglobulin (Fig. 1) where all concentrations of calcium cause excellent precipitation above pH 5.5 to 5.7. Whey is about 3 mM in free calcium, and calcium is sometimes added to milk before it is used for casein manufacture (4).

In the food field, the different behaviors of these two proteins should be considered. Specifically, if β-lactoglobulin were extensively precipitated by a process which leads to a large loss of α-lactalbumin, this process would not be desirable in the nutritional balance of the precipitated protein since α-lactalbumin is the major carrier of sulfur-containing amino acids in milk. The same statement holds for hexametaphosphate. This substance, which is an excellent precipitant for β-lactoglobulin in the pH range of 4 to 5 in acid whey, could increase losses of α-lactalbumin at any pH. The triangles in Fig. 5 show that the minimum of 40% or less remaining soluble can only be attained over a pH range of one unit, from 4.5 to 5.5.

From this, suitability of β-lactoglobulin and α-lactalbumin as models for heat-precipitation of whey becomes questionable. We decided that whey itself must be used even though its

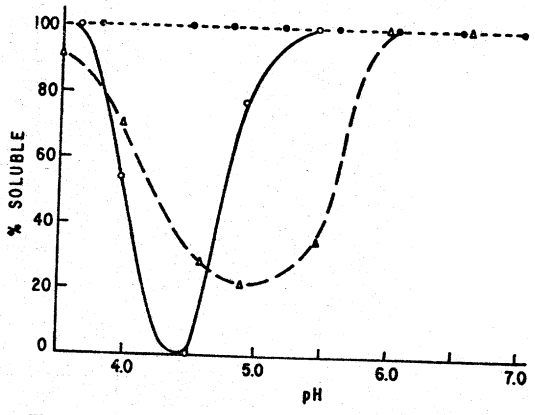

FIG. 5. Effect of solvents on heat-induced precipitation of α-lactalbumin. ○, .1 M NaCl; ●, .07 M $CaCl_2$; △, hexametaphosphate 1 g/100 g protein.

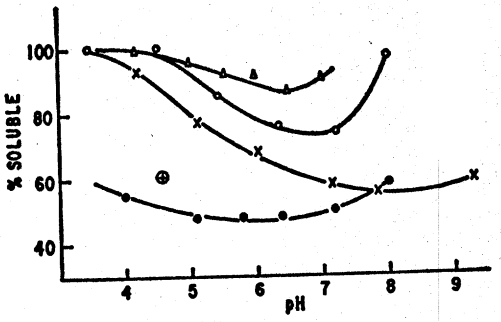

FIG. 6. Precipitation of whole acid whey. ○, whey made .01 M in NaCl; ×, whey with .01 moles $CaCl_2$ added per liter; △, whey with 22 mg/liter HMP added; ●, whey made .006 molar in cysteine • HCl; ⊕, single point — 100 C for .5 h.

protein content is less well defined and lower, therefore more difficult to control and measure. Industrially, the major pollution and/or recovery problem arises with acid whey from cottage cheese manufacture which is much more difficult to dry than sweet or rennet whey. For this reason, precipitation by heat of acid whey was investigated with results in Fig. 6. The open circles show the effect of NaCl added to a concentration of .01 M. The precipitation is essentially unchanged from the whey with no additions. The ×'s record the results with $CaCl_2$ of .01 M. The increase is significant but only at pH values of sweet whey or above; at the natural pH of acid whey (4.4 to 4.7) only a small additional precipitation occurs.

Hexametaphosphate at a concentration of 22 mg/liter seems to prevent heat precipitation; its effect on spray dried whey should be investigated, particularly if maintenance of soluble character is desired for other kinds of food applications. The lower solid line shows the large and significant effect of addition of Cys to whey before heating. These experiments were: Aliquots of acid whey were adjusted to be desired pH, and a quantity of Cys solution was added to give a concentration of .006 M. The pH was readjusted with NaOH if necessary, and the solutions were allowed to stand at 25 C for 1 h before heating. A good precipitation of proteins was obtained over the range of interest, where 50% of the optical absorbance at 280 nm is precipitated between pH 4 and 7.5. This amount is significant if it is compared with the absorbance ratio (⊕, Fig. 6) of a sample of acid whey kept at 100 C for .5 h. A sample of whey made 4% (wt/vol) in trichloroacetic acid and stored at 25 C for 48 h still had 50% of its original optical density, the same amount as remains in solution after treatment with Cys and precipitation.

Disulfide reducing reagent precipitates more optically absorbing material than is precipitated by boiling. It may be that some of the proteose-peptone fraction containing cysteine (or half-cysteine) residues is also made aggregative and precipitated. Cys, when used with β-lactoglobulin dialyzed against the milk system (Fig. 4), has little visible effect on the extent or pH of precipitation. However, in the complex protein system that is whey, addition of reagents capable of causing reduction of disulfide bonds seems highly effective in mediating large-scale and possibly more rapid protein precipitation. Cysteine reduction and subsequent reoxidation by food-approved oxidants might also be a legitimate pretreatment to whole or skim milk which then could be curdled to make cheese supplemented with precipitated whey proteins. This idea is currently being studied with different heating parameters.

Equivalent precipitation may be obtained by heating immediately after cysteine addition or by using β-mercaptoethanol or dithiothreitol as reducing agents. Proteins precipitated by the latter two reagents will not be directly usable in human food, but there may be two practical advantages: (1) Activated sludge sewage disposal beds will rapidly degrade the lactose and small peptides in a deproteinized effluent while whey proteins which resist oxidative degradation can cause foams; and (2) Reverse osmosis to remove salts and lactose undoubtedly will be improved since the slimes which routinely form on semipermeable membranes used to filter whey are proteinaceous. This is not surprising since β-lactoglobulin undergoes an unusually large volume change when denatured (22) so that applied pressure will tend to denature it. This fact alone could account for membrane clogging.

Even if the recovered protein from reduction/heating precipitation could only be sold in the cheapest market, subsequent processing or disposal of the protein-free effluent might be facilitated.

Acknowledgment

The authors wish to thank Mr. S. Elias for supplying the collagen solutions.

Appendix

Correcting measured optical absorbance of supernatants for varying amounts of riboflavin

and nonsedimentable turbidity.

When purified protein solutions are dialyzed against skim milk, a sack containing water is also dialyzed for the same time as an optical reference. This single sample is then heated to 80 C for 15 min along with the samples of the specific series each of which has been adjusted to a different original pH value. Destruction of riboflavin by heat is probably pH-dependent so the reference sample is assumed to have a different riboflavin content than any of the protein samples. In addition, some nonsedimentable turbidity on the protein samples occurs unpredictably and must be corrected. These factors are taken into account:

Spectrophotometric measurements, with the heated water-dialysate in the reference cuvette, are made at 280, 345, and 450 nm.

From the published spectrum of riboflavin (7) it can be determined that the absorbance at 280 nm is 1.84 times as great as that at its peak in the visible (450 nm). Therefore, we subtract from the measured A_{280} the quantity $(1.84 \times A_{450})$, the differential contribution of riboflavin to the 280 nm absorbance with heated dialysate as reference. This correction is small (1 to 2% of A_{280}) but usually positive, indicating that more riboflavin has been destroyed in the dialysate than in the samples (which are generally lower pH).

The contribution of light scattering to the measured A_{280} is estimated. A spectrophotometric reading is taken at 345 nm, the minimum in the riboflavin spectrum. At this wavelength, riboflavin has an absorptivity almost exactly one-half of its absorptivity at the 450 nm maximum. Any absorbance measured at 450 nm is assumed due to riboflavin alone since turbidity is negligible at 450 nm compared to the lower wavelengths. The turbidity then can be represented by $(A_{345} - .5\ A_{450})$; colorless proteins, NPN, etc., do not contribute to A_{345}. The inverse fourth power relationship of Rayleigh scattering (turbidity) predicts that the turbidity at 280 nm is 2.30 times the above quantity, $(1/280^4 \div 1/345^4 = 2.30)$ if the assumption is made that the particles are small enough so that Rayleigh scattering alone accounts for the turbidity. The final equation is:

$$A_{corr} = A_{280} - [2.30\ (A_{345} - 1/2\ A_{450})] - 1.84\ A_{450}$$

where the second term is the 280 nm turbidity correction and the third is the differential contribution of riboflavin.

Protein concentration may be calculated from the extinction coefficient of the protein; or as done for whey, from the ratio of the A_{corr} before and after heating, plotted directly and called percent protein soluble. For purified β-lactoglobulin analyzed at its absorbance maximum of 278 nm, the coefficient of the second term is 2.37; the difference is not significant.

Kjeldahl nitrogen analyses were done on three series of experiments (Table 1). These series cover three separate batches of commercial acid whey; two heated in the presence of Cys and one without added Cys. Nitrogen values were decreased by the amount of N added (as Cys) before being used to calculate

TABLE 1. Corrected optical absorbance and Kjeldahl nitrogen analysis of heated whey supernatants.

Batch	II pH	III A_{corr}	IV Total N mg/ml	V Supernatant N, mg/ml[a]	Ratio Col V/III
1	5.05	7.18	.830	.745	.104
	6.00	5.87	.701	.616	.105
	8.01	8.18	1.004	.919	.112
2	3.98	8.84	1.162	1.086	.123
	4.25	7.30	1.035	.959	.131
	5.03	6.38	.863	.787	.123
	6.00	5.57	.727	.651	.117
	6.96	5.75	.642	.566	.098
	8.03	8.52	1.153	1.077	.126
3[b]	4.32	9.36		1.070	.114
	5.20	8.56		1.021	.119
	5.94	8.59		.935	.109
	6.73	7.70		.842	.109
	7.26	6.81		.748	.110
	7.91	8.32		.951	.114

[a] Values in IV corrected for added cysteine N.
[b] No cysteine added.

ratios in column VI. The internal consistency (SD 2.6%) between the supernatant nitrogen measured and the corrected absorbance calculated is good enough to justify using the correction equation under our narrow set of conditions.

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